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Antisense RNA–Induced Reduction in Murine TIMP Levels Confers Oncogenicity on Swiss 3T3 Cells

RAMA KHOKHA, PAUL WATERHOUSE, SIMCHA YAGEL,*
PEEYUSH K. LALA, CHRISTOPHER M. OVERALL, GILL NORTON,
DAVID T. DENHARDT†‡

Mouse 3T3 cell lines capable of constitutively synthesizing an RNA complementary to the messenger RNA encoding TIMP, tissue inhibitor of metalloproteinases, were constructed by transfection with appropriate plasmid constructs. Many of the lines were down-modulated for TIMP messenger RNA levels and secreted less TIMP into the culture medium. In comparison to noninvasive, nontumorigenic controls, these cells not only were invasive in a human amnion invasion assay, but also were tumorigenic and metastatic in athymic mice. These results indicate that TIMP suppresses oncogenicity, at least in immortal murine 3T3 cells.

A MURINE CDNA CLONE CALLED 16C8 was identified initially by differential screening of a cDNA library with radiolabeled cDNA probes reflecting the mRNA content of quiescent versus serum-stimulated Swiss 3T3 cells (*1*). Synthesis and cytoplasmic abundance of the cognate 0.9-kb mRNA are enhanced by serum, platelet-derived growth factor, double-stranded RNA, and 12-*O*-tetradecanoylphorbol-13-acetate; the product of the mRNA is a secreted glycoprotein (M_r ~29,000) called TIMP, tissue inhibitor of metalloproteinases (*2*).

An inverse correlation between TIMP levels and the invasive potential of murine and human cells has been reported (*3*), implying an important role for TIMP in the control of tumor invasion in vivo. We report here that Swiss 3T3 cells modified genetically to synthesize an RNA (antisense RNA) complementary to the mRNA encoding TIMP are down-modulated for TIMP expression. This down-modulation caused the previously noninvasive cells to become invasive as assessed by an amnion invasion assay. In contrast to control cells, cells producing less TIMP gave rise to tumors and to metastatic lesions 2 to 3 months after subcutaneous or intravenous injection into nude mice.

A full-length cDNA clone encoding mouse TIMP was inserted in both orientations into the Bam HI cloning site of the mammalian expression vector pNMH, and constructs capable of producing sense and antisense transcripts were identified by restriction analysis (*4*). Transcription of the insert is ostensibly driven by the mouse metallothionein-1 promoter; the human growth hormone 3′ untranslated region provides a splice site and termination signals for RNA processing. Plasmid pNMH also has a neomycin resistance gene that confers resistance to G418 sulfate. pNMH-aT, the plasmid designed to produce antisense TIMP RNA, was introduced into Swiss 3T3 cells by coprecipitation with calcium phosphate (*5*), and stable G418-resistant transformants were isolated.

The structure of the integrated plasmid DNA was determined in individual clones by DNA blot analysis (Fig. 1). Four out of ten clones screened had a 2.3-kb Eco RI fragment that is diagnostic of the unrearranged expression cassette; the larger Eco RI fragments of 8.0 kb and 3.1 kb carry portions of the endogenous TIMP gene. LA1 showed a signal at least 100-fold more intense than the signals from the LA6, LA7, and LA8 clones, which appeared to have single-copy inserts. The higher copy number of LA1 could be the result either of gene amplification or of insertion of the plasmid as a concatemer at the site of integration. The differences in the sizes of the Hind III and Bam HI fragments among the various clones indicate that the plasmid had recombined into different sites in the genome and that the cell lines were unique. The G418-resistant MC2 line derived from pNMH-transfected 3T3 cells served as a control.

The extent of stable antisense RNA expression and its effect on the endogenous TIMP mRNA levels was investigated by Northern blot analysis (*1, 6*). Cytoplasmic RNA was extracted from exponentially growing cells, separated by electrophoresis, and blotted onto nitrocellulose. The resulting blot was probed with strand-specific probes to identify sense (Fig. 2B) or antisense (Fig. 2C) TIMP RNA (*7*). One of several controls performed to verify that RNA loading and transfer were uniform is shown in Fig. 2A. An amount of antisense TIMP RNA comparable to mRNAs of moderate abundance was present in the cytoplasm of LA1, but not LA6, LA7, or LA8 cells. Analysis of total cellular RNA by

R. Khokha, P. Waterhouse, G. Norton, D. T. Denhardt, Cancer Research Laboratory, Department of Biochemistry, University of Western Ontario, London, Ontario, Canada N6A 5B7.
S. Yagel and P. K. Lala, Department of Anatomy, University of Western Ontario, London, Ontario, Canada N6A 5B7.
C. M. Overall, Medical Research Council Group in Periodontal Physiology, University of Toronto, Toronto, Ontario, Canada M5S 1A8.

*Present address: Department of Medicine, Hadassah Hebrew University, Jerusalem, Israel.
†Present affiliation: Department of Biological Sciences, Rutgers University, Piscataway, NJ 08855, on leave to the University of Western Ontario.
‡To whom correspondence should be addressed.

Northern blotting also failed to reveal antisense TIMP RNA above background levels in these latter clones. It is possible that the antisense RNA made in the LA6 and LA7 lines annealed to nuclear transcripts of the TIMP gene and was degraded.

Expression of antisense TIMP RNA, presumed to be driven by the metallothionein promoter, appeared to be constitutive since neither Zn^{2+} nor Cd^{2+} was found to influence either sense or antisense TIMP RNA levels (*4*). In addition to background hybridization of the probes to ribosomal RNA, three antisense TIMP RNA species of 0.9, 1.3, and 3.8 kb were consistently detected in LA1 cytoplasm (Fig. 2C). The 1.3-kb RNA species was the size expected for the plasmid construct. The 0.9-kb RNA species may have arisen after hybridization of the 1.3-kb antisense RNA to the 0.9-kb endogenous TIMP mRNA followed by preferential hydrolysis of the single-stranded RNA overhangs. The 3.8-kb species may represent a transcript of the complete plasmid.

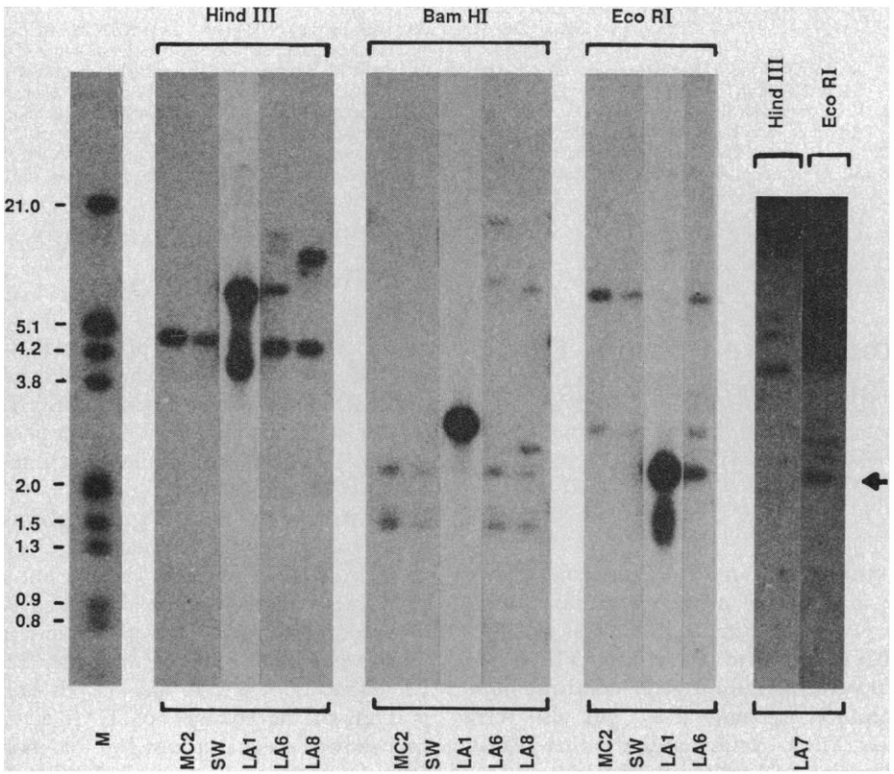

Fig. 1. Southern blot analysis of the genomic DNA in transfected cell lines. LA1, LA6, LA7, and LA8 were produced by transfection of Swiss 3T3 cells with the plasmid pNMH-aT, which carries the TIMP cDNA in the antisense orientation (*4*). MC2 is a G418-resistant line transfected with pNMH. SW is the original Swiss 3T3 G418-sensitive recipient. Ten micrograms of DNA was restricted with the indicated enzyme, and the resulting DNA was separated by electrophoresis in a 1% agarose gel; after blotting onto nitrocellulose, the filter was probed with a TIMP cDNA insert labeled by nick translation with the use of DNA polymerase I and [α-^{32}P]dNTP (*6*). The arrow indicates the 2.3-kb Eco RI fragment bearing the complete, unrearranged expression cassette containing the TIMP insert. The analysis of the LA7 DNA was performed in a separate experiment. The exposure time for LA1 was 30- to 40-fold less than that for the other cell lines. Markers (M) were derived from λDNA cleaved with Eco RI plus Hind III and end-labeled with the Klenow polymerase and [α-^{32}P]dATP.

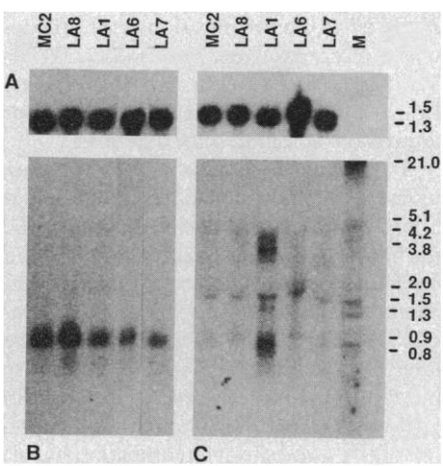

Fig. 2. Northern blot analysis of the RNA in transfected cell lines. Cytoplasmic RNA (10 μg) was denatured in formaldehyde/formamide, separated by electrophoresis in the presence of HCHO on a 1.1% agarose gel, and transferred to nitrocellulose (*7*). The blot was hybridized successively with the following probes: (**A**) pBR322 carrying a glyceraldehyde-3-phosphate dehydrogenase cDNA insert, (**B**) a probe specific for TIMP mRNA, and (**C**) a probe specific for antisense TIMP RNA. The probe in (A) was labeled by nick translation as described in Fig. 1; the ^{32}P-labeled probes in (B) and (C) were prepared as described (*7*). In comparison with the control MC2 line, TIMP mRNA levels in LA1, LA6, and LA7 were 45%, 71%, and 33%, respectively; these values, each with standard deviations of $\pm$3%, were derived by averaging a dozen scans, made with an LKB Ultroscan laser densitometer, of autoradiograms in the linear range of exposure. λ Phage DNA markers are as in Fig. 1. Cell lines are the same as in Fig. 1. Exposure times for the blots in (B) and (C) are comparable.

A significant reduction in the steady-state abundance of TIMP mRNA was observed in the LA1, LA6, and LA7 clones, whereas LA8 had TIMP mRNA levels comparable to the control Swiss 3T3 and MC2 cell lines. The level of TIMP activity in the conditioned culture media of these cell lines was assessed by an in vitro assay for collagenase inhibitor activity (*8*). Data in Table 1 show that the TIMP activity observed in media conditioned by cell lines down-modulated for TIMP mRNA levels was approximately half that seen in the control clones. The close quantitative relation between the extent of down-regulation of TIMP mRNA and secreted TIMP activity provides strong evidence that the inhibitor measured in this assay was indeed TIMP. The ability of LA1 to produce as much TIMP as LA6 and LA7 suggests that the presence of antisense RNA in the cytoplasm of this line was not having a significant additional negative effect on TIMP production, supporting the thesis that the antisense RNA is exerting its effect in the nucleus. TIMP was not down-modulated in LA8, perhaps because the expression cassette was transcriptionally inactive.

The cells with lowered TIMP mRNA levels (LA1, LA6, and LA7) exhibited subtle alterations in morphology and growth characteristics; they were more refractile and their volume (measured with a Coulter counter) was about 25% smaller than control cells. The density that these cells reached in monolayer cultures at confluence was almost twice that achieved by LA8 and the control Swiss 3T3 and MC2 cells, and they showed a decreased tendency to become quiescent in low serum. Thus after incubation in medium containing 1% fetal bovine serum for 1 week, some 5% of the cell nuclei could still be labeled by an 18-hour pulse of [^{3}H]thymidine; under equivalent conditions, only 1% of the control cells became labeled (*9*). However, by most conventional in vitro criteria the cells down-modulated for TIMP expression were not transformed: they were susceptible to contact inhibition, they ceased DNA synthesis when maintained in 0.5% serum, and the small colonies that formed in soft agar at an efficiency of 25

to 45% did not grow to contain more than 16 to 32 cells (*10*). Cells from the parental and control lines rarely succeeded in dividing even once.

The invasive ability of these cell lines was assessed by an amnion invasion assay (*11*). [^{125}I]Iododeoxyuridine-labeled cells were placed on amnion basement membranes; invasion was quantified by the radioactivity associated with the membranes 72 hours later (Fig. 3). LA1 possessed the highest invasive potential, with some 2.3% of the total radioactivity becoming associated with the amnion basement membrane. The LA6 and LA7 clones were slightly less invasive, whereas LA8, MC2, and Swiss 3T3 cells were essentially noninvasive. The highly invasive B16F10 mouse melanoma cells (*12*), used as a positive control, showed an invasion index of 3.1%.

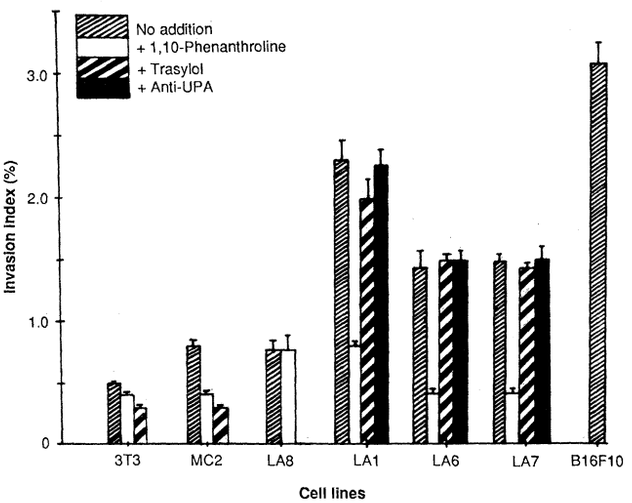

Fig. 3. Amnion invasion by cell lines down-regulated for TIMP expression. The amnion membranes were fastened to Teflon rings (invasion chambers), placed onto silicone rubber supports in culture wells with the stromal aspect facing down, and amniocytes removed with ammonium hydroxide (*11*). Aliquots (200 μl) of medium (Dulbecco's modified Eagle's medium with 10% fetal calf serum), with or without the proteinase inhibitors or antibodies to be tested, were added to the upper chambers and, after incubating the plates at room temperature for 1 hour, 1.5×10^5 [^{125}I]iododeoxyuridine-labeled cells in 500 μl were added to the 200 μl in the upper chamber. The concentrations of the inhibitors were 10 μg of 1,10-phenanthroline per milliliter, 200 units of Trasylol per milliliter, and 1% of affinity-purified rabbit antibody to mouse urokinase-type plasminogen activator. The plates were incubated at 37°C in a water-saturated 5% CO_2 atmosphere for 72 hours, then the medium was removed and surface-associated cells eliminated by lysis followed by scraping and washing of the membrane surface with phosphate-buffered saline. The radioactivity associated with the amnion, as well as that of the medium and washings, was expressed as percent of the total radioactivity, determined by summing the radioactivity in the individual compartments. The invasion index is the percent of radioactivity retained in the amnion membrane, and each bar in the figure represents the mean of three measurements ± the standard error.

Experiments were performed with inhibitors (Fig. 3) of various proteinases to characterize the responsible enzyme or enzymes. Invasion of the amnion by LA1, LA6, and LA7 cells was prevented by the chelating agent 1,10-phenanthroline, firmly implicating metalloproteinases in the process. In contrast, invasion was not blocked by antisera against either plasminogen (*13*) or the urokinase-type plasminogen activator (anti-UPA). Trasylol (aprotinin), a serine protease inhibitor, was also ineffective in blocking invasion by these cell lines, suggesting that their invasiveness was not dependent on the generation of plasmin to activate procollagenase. Medium from a cell line (16S1) that was up-modulated for TIMP expression effectively reversed the invasiveness of LA1.

We investigated the tumorigenic and metastatic potential of the TIMP-down-modulated and control lines in athymic (nude) mice. The nude mouse system was chosen in order to eliminate the possibility of immune rejection of the transfected 3T3 cells (*14*). In an experimental metastasis assay, injection of LA1 cells into the tail veins of four mice produced lung tumors in three of the mice within 3 months. Subcutaneous inoculation into the chest wall of LA1 or LA7 cells led in each case to tumor formation in nine of nine mice within 2 months (Table 2). Macroscopic lung metastases, evidence of spontaneous metastasis, were evident in the lungs of the moribund animals, which were killed

Table 1. Tissue inhibitor of metalloproteinase (TIMP) activity in culture media conditioned for 24 or 72 hours by Swiss 3T3 cell lines transfected with plasmid constructs encoding TIMP in the antisense orientation. Data from a representative experiment ($\bar{x} \pm$ SD, $n = 3$) are shown; in two independent repetitions comparable results were obtained. The percentage change in TIMP expression in experimental cells compared with control MC2 cells is shown in parentheses. Cells were plated at 9×10^5 cells per 100-mm dish and incubated in Dulbecco's modified Eagle's medium supplemented with 10% (w/v) fetal bovine serum and antibiotics. At confluence the cultures were maintained in serum-free medium supplemented with insulin, transferrin, and selenium for 3 days, and portions of the conditioned medium were harvested daily. TIMP activity in the conditioned medium was determined from the inhibition of a standard amount of purified active porcine gingival explant collagenase (*8*). Equal portions of diluted medium were preincubated with 400 U collagenase for 30 min at 22°C before addition of ~6 ng [^{14}C]glycine-labeled collagen (specific activity 3.5×10^8 dpm/mg). The reactions were then incubated at 27°C for 19 hours. TIMP was quantified following resolution of the reaction products by SDS-polyacrylamide gel electrophoresis and laser densitometry of the collagenase-specific 3/4-collagen fragments from the fluorographs in the linear range of the assay; customized software was used for integration of the peaks generated. The inhibitor assay was sensitive down to ~1 pg TIMP, as determined by calibration of the assay with purified human fibroblast TIMP (*19*). One unit of TIMP inhibits 2 units of collagenase by 50%; 1 unit of collagenase degrades 1 pg of soluble type I collagen per hour at 27°C. For all assays substrate controls and collagenase assays were performed; no nonspecific degradation was observed and no active collagenase was detected.

Cell lines	TIMP activity (units per 10^6 cells)	
	24 hours in medium	72 hours in medium
Controls		
Swiss 3T3	656 ± 100	690 ± 37
MC2	650 ± 140	600 ± 140
LA8	765 ± 110 (120%)	640 ± 120 (110%)
Down-modulated		
LA1	440 ± 12 (68%)	315 ± 33 (53%)
LA6	390 ± 60 (60%)	340 ± 33 (57%)
LA7	330 ± 68 (51%)	280 ± 72 (47%)

Table 2. Tumor formation and metastasis in nude mice. Four- to 6-week-old BALB/c nu/nu female mice were injected with 4×10^6 cells into the right anterior lateral thoracic wall. The mice were killed after the tumor reached a diameter of some 2 cm (about 3 months). In all cases the chest wall was observed to be intact. All internal organs were examined for visible metastatic foci. Primary tumors and lungs were fixed in Bouin's fixative for subsequent histological examination. Peribronchial metastases of the lung tissue were the predominant lesions; there were no obvious differences in the number or extent of metastases from the LA1 and LA7 lines. LA6 was not investigated because it showed a tendency to revert in the in vitro studies.

Cells injected	No. of mice with primary tumor per no. of mice injected	No. of mice with metastases per no. of mice injected
LA1	9/9	9/9
LA7	9/9	9/9
LA8	0/5	—
Swiss	0/5	—
MC2	0/5	—

after 3 months. Cells derived from the primary tumors or from tumor-bearing lung tissue gave rise to G418-resistant lines, indicating that the tumors had arisen from the injected cells. Mice inoculated with the control Swiss 3T3 or MC2 lines showed no sign of tumor formation even after 8 to 9 months. The LA8 cell line, which behaves like the control cell lines with respect to TIMP mRNA and protein production, also did not form tumors. Since it takes somewhat longer for tumors to develop from these cells than is the case for fully transformed, malignant cells, we suspect that further changes in gene expression are required in addition to the down-regulation of TIMP production. Alternatively, the increased metalloproteinase activity, presumed to result from decreased TIMP expression, may affect the immediate environment of the already immortal cell in vivo in such a way as to stimulate its proliferation and foster tumor formation.

We have shown above that in cells engineered to synthesize antisense TIMP RNA the levels of TIMP and its mRNA are effectively down-modulated. Reduction in expression of this one gene reproducibly conferred tumor-forming and metastatic behavior on normal Swiss 3T3 cells; this appears to certify TIMP as a tumor suppressor. We argue that only one gene (TIMP) is directly affected because our 16C8 probe (TIMP) detects only a single gene and a transcript of homogeneous size. Since 1,10-phenanthroline was able to abrogate invasion, it is reasonable to attribute the invasive behavior of these cells to an increased metalloproteinase activity. Because of the high affinity of TIMP for metalloproteinases ($K_a < 10^{-9}$), small changes in the amount of TIMP produced can have a dramatic effect on the level of metalloproteinase activity (*15*). Neutral metalloproteinases such as stromelysin and gelatinase that degrade components of the basement membrane are known to be produced by many cell types, and their expression has been found by a number of investigators to correlate with the metastatic potential of some tumor lines (*16*). Schultz *et al.* (*17*) have observed that recombinant TIMP exhibits an antimetastatic effect in vivo. Other research has also suggested an important role for proteinase inhibitors in controlling the tumorigenic process (*18*).

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